a protonatable side chain in E2P-like conformations might allow shuttling of protons across the membrane. We therefore evaluated the role of three key carboxylates, E336 (TM4), E788 (TM5), and D935 (TM8), by mutating C113Y *Xenopus* pumps. Outward Na/K-pump current was practically abolished in E336Q(C113Y) or E336C(C113Y) pumps, but nonstoichiometric inward current at zero Ko and Nao (replaced by TMAo) was little altered, and was greatly augmented in high Nao. E788C(C113Y) pumps, on the other hand, like parent C113Y pumps, generated robust stoichiometric outward Na/K transport currents (voltage dependent in Nao, but not in TMAo), and little nonstoichiometric inward current in high Nao; but nonstoichiometric current in TMAo was diminished ~3-fold compared to parent C113Y pumps. In contrast, D935N(C113Y) pumps generated stoichiometric outward Na/K transport currents with altered voltage dependence that was similar in Nao or in TMAo, but inward nonstoichiometric current was nearly absent both in Nao and in TMAo, and was not augmented by lowering pH to 6 either in Nao and in TMAo. The D935 carboxylate thus seems uniquely required for the nonstoichiometric inward flow of protons through the Na/K-pump. [NIH HL36783].

2512-Pos Board B498

Single-Molecule Studies of the Na^+/K^+-ATPase

Promod R. Pratap, Gregor Heiss, Martin Sikor, Don C. Lamb, Max Burnett.

The Na^+/K^+-ATPase, a membrane-associated ion-motive ATPase, uses energy from the hydrolysis of ATP to move Na^+ out of and K^+ into cells. We have labeled ATPase isolated from duck supraorbital salt glands with Cy3-maleimide (Cy3-ATPase). We have previously found that the fluorescence of Cy3-ATPase decreases in the presence of ATP (*Biochim Biophys Acta* 2009; **1794**:1549-1557). We found there that the kinetics of this ATP decrease exhibited negative cooperativity. To determine whether this behavior is due either to interaction between protomers or is an intrinsic property of monomers, we examined the fluorescence of the labeled enzyme solubilized in the nonionic detergent $C_{12}E_8$ in the absence and presence of varying concentrations of ATP using single-molecule total internal reflection spectroscopy (SM-TIRF). We found that: (i) our Cy3-maleimide labeling protocol yielded a significant fraction of singly-labeled protein; and (ii) even with solubilization, a significant fraction of the protein exhibited aggregation. A preliminary analysis of the data from single (non-aggregating) molecules using a hidden Markov model (HMM) suggests a difference in the single-molecule dynamics of the enzyme in the presence and absence of ATP. The implication of these observations will be discussed.

2513-Pos Board B499

A Novel Endogenous Cardiotonic Hormone from Mammalian Muscle and Kidney

Judith A. Heiny, Leonid N. Koikov, Frederic Mandel,
Tatiana L. Radzyuykevich, Stephen F. Macha.

The cardiotonic receptor on the Na,K-ATPase and an endogenous ligand play a physiological role in regulating its enzyme activity in mammalian skeletal muscle (Radzyukevich et al. 2009. *PNAS* 106(8):2565-70). The goal of this study was to develop an approach to identify the endogenous ligand(s). The regulation is preserved *in vitro* and crude muscle extracts retain active factor(s). Therefore, we used muscle as a source of concentrated bound ligand, in combination with a batch affinity purification method which uses the receptor itself to capture its ligands. We applied affinity extraction with a purified Na,K-ATPase to a MeOH extract from skeletal muscle. Ligands were bound to the receptor in Mg^{2+}-containing buffer and released in EDTA. The efficiency of ligand binding was confirmed and optimized using ^{3}H-ouabain. Binding of Mg^{2+} to a single Mg binding site enhances binding at the cardiotonic receptor site by a factor of 215, and thereby favors specific over potentially non-specific ligand binding. The resulting compounds were desalted on hydrophobic SPE cartridge and separated on HPLC with a C18 column. One of the fractions from muscle showed a single peak in MS (m/z 381 M + Na^+). This compound is distinct from ouabain (m/z 606), ouabagenin (m/z 460), marinobufagenin (m/z 423) and other known cardiotonic steroids. We obtained the same factor from pig kidney, plus 3 additional compounds. These results demonstrate that at least one novel cardiotonic hormone is common to different mammalian species and tissues. Given the broad scientific and therapeutic implications of the cardiotonic hormones, our current goal is to accumulate a sufficient amount of this compound for structural identification by cryo NMR. This goal is feasible using a scaled up procedure which yields nanogram amounts (in ouabain equivalent units) per run.

2514-Pos Board B500

Na Pump E960 Site is Critical for the Interaction with Phospholemman

Mounir Khafaga, Julie Bossuyt, Joseph C. Li, Linda L. Lee,
Jeffrey H. Elliott, Sanda Despa, Donald M. Bers.

Phospholemman (PLM), a FXYD family member, critically modulates Na pump (NKA) and thus is important in Na and Ca regulation in cardiac myocytes. PLM inhibits NKA by reducing its apparent Na affinity, effect relieved by PLM phosphorylation. However, the sites responsible for the NKA-PLM interaction are unknown. Based on the recent NKA crystal structure with the associated FXYDs, we constructed single-site CFP-NKAα1 mutants with alanine substitution at F956, E960, L964 and F967 sites. Mutated CFP-NKAα1 and wild-type (WT) PLM-YFP were co-expressed in HEK cells and their interaction was assessed using fluorescence resonance energy transfer (FRET). FRET efficiency was determined as the relative increase in donor fluorescence (ΔF_{CFP}) upon acceptor photobleaching. The NKA-PLM FRET was not altered by the F956A and F967A mutations, was significantly reduced for L964A (ΔF_{CFP} =6 ± 1% *vs.* 17.9 ± 2% for WT-NKA) and was abolished for E960A (ΔF_{CFP} =0.1 ± 1%). Alanine mutation of the PLM site F48, shown by X-ray crystallography to be within interaction distance of NKA E960, eliminated the FRET with WT NKA (ΔF_{CFP}=0.2 ± 4%). Mutation of the same site to cysteine, as in some FXYD proteins, did not affect the NKA-PLM FRET (ΔF_{CFP}=18.5 ± 1.7%). To determine whether PLM affects the function of the E960A NKA mutant, we measured the NKA-mediated Na-extrusion as a function of $[Na]_i$ in a cell line that stably expresses E960A-NKA, with and without WT PLM. The apparent NKA-E960A affinity for $[Na]_i$ was similar in the absence (K_d=9.7 ± 1.0 mM) and in the presence (K_d=8.9 ± 1.7 mM) of WT-PLM and was not significantly affected by forskolin-induced PLM phosphorylation. In contrast, WT-PLM reduced the Na-affinity of WT-NKA (K_d increased from 9.3 ± 1.6 to 11.5 ± 1.9 mM) and PLM phosphorylation lowered the K_d to 9.8 ± 1.8 mM. Thus, our results demonstrate that the E960-F48 interaction is critical for the PLM-NKA association.

2515-Pos Board B501

Uncoupled Inward Currents through Native Na/K Pumps in Guinea Pig Ventricular Myocytes

Camila Zugarramurdi, Juan J. Ferreira, **Pablo Artigas**.

The Na/K pump is a P-type ATPase that maintains essential electrochemical gradients for Na^+ and K^+ across the plasmalemma of animal cells. Within its transmembrane domains the pump presents 3 ion-binding sites, two of which can bind Na^+ or K^+ (shared sites) and another that exclusively binds Na^+ . The mechanisms by which different ions are selected by each site are not fully understood.

Electrophysiological studies from several laboratories investigating the function of Na/K pumps in *Xenopus* oocytes have shown that, without external Na^+ or K^+, the pump passively imports protons and possibly guanidinium$^+$ and its derivatives. It is thought that both protons and guanidinium-derivatives are transported through the Na^+ -exclusive site when the shared sites are empty. Because ion-binding sites of Na/K pumps across the animal kingdom are conserved, it is puzzling that these currents have not been reported in classical preparations where native Na/K pumps have been studied under voltage clamp. Here, we describe these uncoupled inward currents through the native Na/K pumps of Guinea pig ventricular myocytes, demonstrating they are not an artifact of the oocyte system.

Under whole-cell patch-clamp with internal conditions promoting maximal Na/K pump phosphorylation (50 mM Na^+_i, 5 mM MgATP), cardiotonic steroid-sensitive inward currents (I_{unc}) were not observed in 150 mM Na^+_o (at all pH_o) or in 150 NMG^+_o (at pH_o =7.4). In contrast, at negative voltages, large currents I_{unc}=-1,7 ± 0,26 pA/pF (at −180 mV, n=10) were observed in NMG^+_o solutions with pH_o = 6. Also consistent with observations reported in oocytes, inward currents I_{unc} = −1,08 ± 0,14 pA/pF (n=14) were observed in 150 mM guanidinium^+_o (pH_o =7.4). The effects of other Na/K pump ligands on I_{unc} are underway. Financed by TTUHSC SABR and AHA BGIA2140172.

2516-Pos Board B502

The Sodium Pump is Confined in a Phosphoenzyme Form by Lead(II) Ions

Gianluca Bartolommei, Elisa Gramigni, Francesco Tadini-Buoninsegni,
Giacomo Santini, Maria Rosa Moncelli.

Steady-state fluorescence spectroscopy was used to investigate the effect of Pb^{2+} ions on the Na^+,K^+-ATPase. Experiments were performed by using the electrochromic styryl dye RH421 (1, 2) to characterize in detail the effect of Pb^{2+} ions on the Na-pump and to pinpoint the reaction step(s) of the enzymatic cycle at which the heavy-metal ions provoke their action.

We recently found that Pb^{2+} ions completely inhibit enzyme activity at concentrations above 10 μM (K_I = 0.5 μM (3)). It is now shown that Pb^{2+} ions can bind reversibly to the protein and do not affect the Na^+ and K^+ binding affinities in the E_1 and P-E_2 conformations of the enzyme. This indicate that Pb^{2+} binding to the protein does not block the access pathway to ion binding sites. We also found that lead(II) favors binding of one H^+ to the P-E_2 conformation in the absence of K^+. A model scheme is proposed that accounts for the experimental